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(54) Abstract Title
Detecting temperature or metabolic activity of cells

(57) The temperature of a cell is obtained by
(a) placing a cell on a metabolic probe comprising a solid substrate containing a temperature-sensitive fluorophore embedded in a polymer, wherein when the fluorophore is excited with ultraviolet, visible or infrared light it emits a detectable fluorescent signal; and wherein when a living cell is placed on the metabolic probe, the fluorescent signal is effected by the metabolic rate of the cell;
(b) exciting the fluorophore with ultraviolet, visible, or infrared light; and
(c) detecting the fluorescent signal, wherein the intensity of fluorescent signal detected is indicative of the temperature of the cell.

The measured temperature may be used to determine the metabolic activity of the cell and monitor any abnormality. Polymer is e.g. PMMA and the fluorophore may be $Eu(TTA)_3$.

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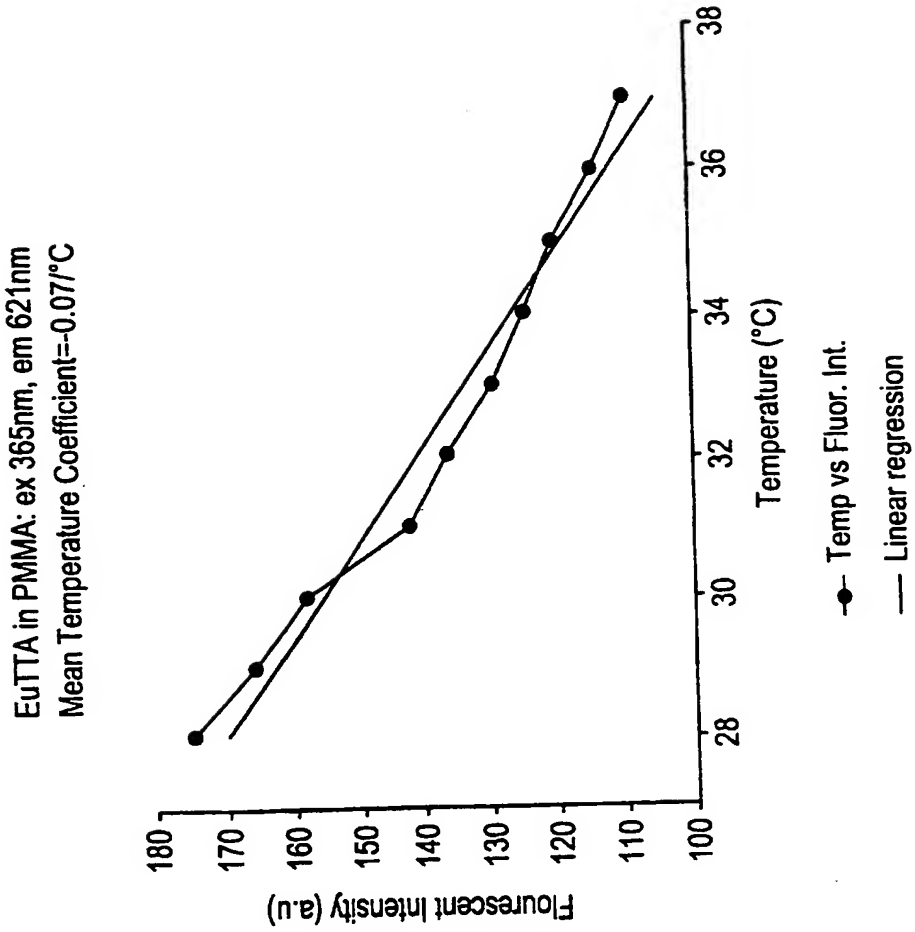
FIG. 1


EuTTA in PMMA: ex 365nm, em 621nm
Mean Temperature Coefficient=-0.07/°C
Fluorescent Intensity (a.u)
180
170
160
150
140
130
120
110
100
28
30
32
34
36
38
Temperature (°C)
Temp vs Fluor. Int.
Linear regression

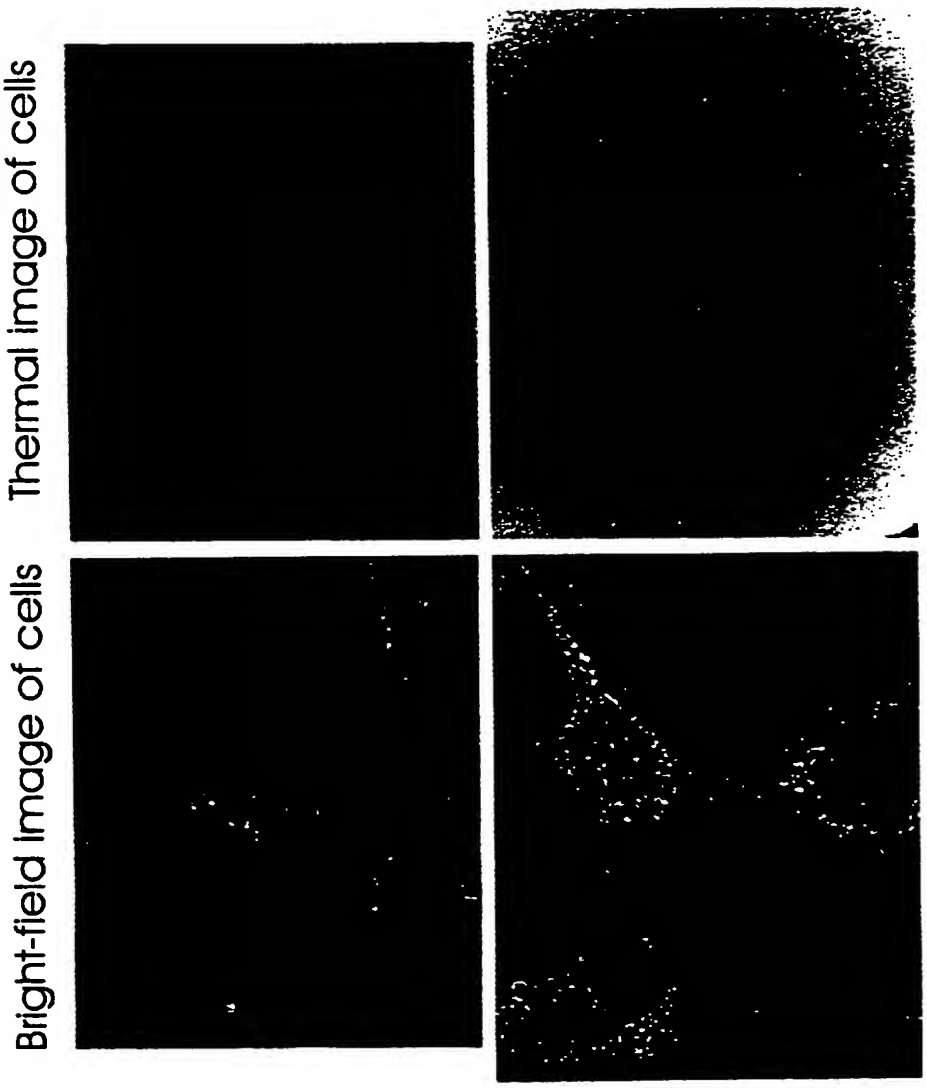


FIG.2B

FIG.2D

FIG.2A

FIG.2C

A METHOD FOR DETERMINING THE TEMPERATURE OF A SINGLE CELL IN A CELL SAMPLE OR TISSUE BIOPSY, AND METHODS OF USE THEREOF

FIELD OF THE INVENTION

The invention relates generally to a novel method of determining the temperature of a single cell, or group of cells, in a tissue sample. Methods of employing this method as a diagnostic are also included.

BACKGROUND OF THE INVENTION

Living cells require a stable rate of metabolism. Therefore in both cells and in the organisms containing the cells such regulation is under stringent control. Approximately 50% of all energy in carbohydrates is converted into ATP and the rest is released as heat [Alberts *et al., Molecular Biology of the Cell,* (1989)]. The ATP, in turn, is used during growth for biosynthesis, and otherwise is used for work and the energy is released as heat. The release of this heat is used to warm the body in the form of temperature. Failure to maintain a stable metabolic level on an organismal level is a pathology referred to as fever - an increase in the temperature of an organism. Variations in metabolic levels are seen in a variety of pathological states including during bacterial or viral infections, in cancer, and in the general wasting away found during sepsis or cachexia.

The temperature of an organism is often taken as being indicative of the health of that organism. Even slight deviations in temperature may indicate a pathology. However the temperature of an individual cell cannot be assayed due to the limitation of current technology. Thus, it is not yet known to what extent cellular temperature varies with time within a cell or between two different cells. However, based on studies of large populations of cells there are good grounds to believe that some pathological states may be detectable by measuring temperature at the level of single cells.

Cellular metabolism may be regarded as the balance sheet for all of the enzymatic reactions occurring in the cell. Within this context, the next exothermic activity is seen as black body radiation, *i.e.*, the heat that we assay when we measure the temperature of the cell. Prior studies of biological metabolic activity have used calorimetry which is applied at the level of an entire organism, or at the very least an individual organ. Microcalorimetry can be applied to as few as 10 cells. However, there are severe limitations in the current technology limit resolving the heat production from single cells [Kemp, *Thermal and Energetic Studies of Cellular Biological Systems*, A.M. James, ed. (Bristol: Wright), pp. 147-166 (1987)]. The most sensitive technique applied to measuring metabolism in tumor cells is the Cartesian diver which can resolve hundreds of cells [Lutton and Kopac, *Cancer Res.*, 31:1564-1569 (1971)]. This technique, however, is still too crude to resolve a heterogeneous population of cells, and it requires dissociation of the cells. Much of normal cell physiology is a dynamic process that requires cellular interaction in a three dimensional matrix. Many cellular activities are modified by cell-to-cell contact. All of this is lost when cells are dissociated. Recently techniques have been developed to measure metabolites such as ATP, glucose and lactate in living cells [Hossman *et al.*, *Acta Neuropathologica*, 69:139-147 (1986); Okada *et al.*, *Journal of Neurosurgery*, 77:917-926 (1992)]. These techniques utilize photographic film [Hossman *et al.*, 1986, *supra*; Okada *et al.*, 1992, *supra*] or photon-counting cameras [Tamulevicius and Streffer, *British Journal of Cancer*, 72:1102-1112 (1995)] and have demonstrated considerable heterogeneity in metabolism in tumors when assayed with resolution on a millimeter spatial resolution.

Unfortunately, presently a number of important issues cannot be addressed because the metabolism of an individual cell cannot be determined. For example, we cannot presently study heterogenous populations of cells and resolve the activity of individual cells rather than the average of the mix. For example, a tumor is made of cancerous cells, normal cells, and infiltrating immune cells each of which is metabolizing at very different rates. In this case the measurement of the average metabolism of the tumor may not reflect the actual metabolism of the individual cells.

In most cells aerobic respiration is responsible for almost all of the production of ATP with anaerobic glycolysis accounting for the remainder. It was noted over fifty years ago that anaerobic respiration is substantially increased in Ascites tumor cells relative to non-tumor cells [Warburg *et al., The Metabolism of Tumors*, ed. O. Warburg, Constable & Company LTD., London, pp. 129-170 (1930a); Warburg *et al., The Metabolism of Tumors*, ed. O. Warburg, Constable & Company LTD., London, pp. 254-265 (1930b)]. This led to the hypothesis that aerobic respiration was damaged in tumor cells [Warburg, *Science*, 123:309-314 (1956)]. As more has been learned about metabolism in the intervening years it was found that aerobic respiration is normal in tumor cells, but anaerobic respiration is increased. The mechanism responsible for this increase in anaerobic respiration is not presently understood. One major limiting factor in learning the mechanism is the inability to assay the relative metabolic levels of individual cells. As mentioned above, one particular problem is that tumors consist of a mix of normal, malignant, and immune cells. Current technology only allows the measurement of the average metabolism of this mixed population of cells. A second problem is the considerable heterogeneity even within the tumor cells. For example in tumors, oxygenation is often rate limiting for tumor growth [Kallinowski *et al., J. Cel. Physiol.*, 138:183-191 (1989)]. Thus, in rapidly growing tumors growth is limited by angiogenesis, the growth of new blood vessels for the delivery of oxygen and nutrients.

Chemotherapy is a power tool that is used to fight tumors. However, tumor cells frequently develop resistance to the chemotherapeutics. This resistance is observed as a decreased sensitivity to a broad spectrum of chemotherapeutic agents and such cells have been labeled multi-drug resistant [Simon *et al., Proc. Natl. Acad. Sci. USA*, 91:3497-3504 (1994)]. Although these cells were originally viewed as "super cells" capable of withstanding any therapeutic challenge there is now growing evidence that multi-drug resistant cells escape chemotherapy by behaving more like normal cells; and in some ways these cells appear to have undergone a "reverse" transformation in their properties [Biedler *et al., Cancer & Metastasis Reviews*, 13:191-207 (1994)]. Once chemotherapeutic drugs are removed, these cells resume their aggressive

malignant properties. It is not known what happens to the metabolism of multi-drug resistant cells during this period. Indirect results indicate that the level of anaerobic respiration in these cells have returned to the levels seen in non-transformed cells [Miccadei *et al., Oncology Research,* 8:27-35 (1996)]. Therefore there is a need to measure the metabolism of individual cells that are multi-drug resistant in order to diagnose when tumors are shifting from their quiescent multi-drug resistant phase into a more malignant stage. Further there is a need to provide a method of measuring the temperature of individual cells to determine the metabolic changes that occur in either normal or tumor cells. In addition, there is a need of a methodology for measuring the temperature of a cell from a fresh biopsy of living tissue in order to rapidly diagnose the tissue for the presence of tumor cells.

The emission of almost all fluorophores is affected by temperature, however, the use of fluorophores that are particularly sensitive to temperature was first used as a technique for calibrating temperature by Kolodner et al., [*Appl. Phys. Lett.* 40:782-784 (1982); *Appl. Phys. Lett.* 42: 782-784, (1983)]. Kolodner used a fluorophore, $Eu(TTA)_3$ embedded in a polymer, PMMA, to achieve temperature resolutions of 0.07°K and of 10 μM spatial resolution. This temperature-sensitive fluorophore thus, could be used to quantify temperatures.

A variety of fluorophores, including $Eu(TTA)_3$ have been solubilized into various solvents and polymers and used to "paint" airplane wings in the laboratory of John P. Sullivan (Pursue University, see Table 1 modified from [Campbell *et al.,* 1994, *supra*]). The change in fluorescence can be used to monitor the temperature changes of the airplane wing in a wind tunnel, for example. To date, temperature-sensitive fluorophores have been used in integrated circuit diagnostics [Kolodner *et al., Appl. Phys. Lett.,* 42:117-119 (1983)], to detect boundary layer transition on a two-dimensional wing, and to visualize the interaction between the leading edge vortices and the surface of a delta wing [Campbell *et al.,* in sixth Intern. Symp. on Applications of Laser Techniques to Fluid Mechanics, Lisbon, Portugal (1992); Campbell *et al., Temperature Measurement Using Fluorescent Molecules,* Abstract

(1992); Campbell *et al., Temperature Sensitive Fluorescent Paint Systems,* 18:94-2483 (1994); Hamner *et al., A Scanning Laser System for Temperature and Pressure Sensitive Paint.* 32:94-0728 (1994)].

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

SUMMARY OF THE INVENTION

The present invention, in its broadest embodiment, provides a means of measuring the temperature of a cell by using a temperature-sensitive fluorophore. The present invention employs a metabolic probe which comprises a solid substrate containing a temperature-sensitive fluorophore embedded in a polymer. When the fluorophore is excited with the appropriate ultraviolet, visible, or infrared light, the fluorophore emits a detectable fluorescent signal. When a living cell is placed on the metabolic probe the intensity of the fluorescent signal is effected by the metabolic rate of the cell.

Preferably the metabolic probe contains a fluorophore which can emit a stable fluorescent signal while embedded in a polymer adjacent to an aqueous solution for at least one hour upon excitation with ultraviolet light at 37°C, at about pH 7.5. In a particular embodiment of the invention, a cell placed on the metabolic probe, causes a 2.5% change in intensity of the fluorescent signal per degree Celsius increase in temperature.

In one embodiment the temperature-sensitive fluorophore contained by the metabolic probe is $Eu(TTA)_3$. In another embodiment the polymer of the metabolic probe is poly(methyl methacrylate). In a preferred embodiment the polymer is poly(methyl methacrylate) and the temperature-sensitive fluorophore is $Eu(TTA)_3$. The solid substrate of the present invention can be made of any inert material but is preferably a

glass or a plastic. In a more preferred embodiment of this type, the solid substrate is a glass cover slip.

The present invention provides a method of detecting the temperature of a cell. One such embodiment comprises placing a cell on a metabolic probe which comprises a solid substrate containing a temperature-sensitive fluorophore embedded in a polymer. When the fluorophore is excited with the appropriate ultraviolet, visible or infrared light it emits a detectable fluorescent signal. When a living cell is placed on the metabolic probe, the intensity of the fluorescent signal is effected by the temperature of the cell.

In one specific embodiment of this method, the polymer is a non-aromatic synthetic polymer. In one particular embodiment of this method, upon excitation with ultraviolet light the fluorophore can emit a stable fluorescent signal for at least one hour at 37°C, pH 7.4.

The present invention also includes a method of detecting the metabolic activity of a cell. One such embodiment comprises detecting the temperature of a cell by placing the cell on a metabolic probe of the present invention, exciting the fluorophore of the probe with the appropriate ultraviolet, visible, or infrared light, and detecting the fluorescent signal, wherein the intensity of the fluorescent signal detected is indicative of the temperature of the cell. The temperature measured is then correlated with the metabolic activity of the cell. In a preferred embodiment of this type the cell is a tumor cell.

The present invention also includes a method of detecting the presence of a tumor cell in a living tissue comprising detecting the temperature of the cell by placing the cell on a metabolic probe of the present invention (as described above), exciting the fluorophore of the metabolic probe with the appropriate ultraviolet, visible, or infrared light, and detecting the fluorescent signal. The intensity of the fluorescent signal detected can be correlated to the temperature of the cell. The metabolism of tumor

cells is known to be significantly higher than that of non-transformed cells. The present invention allows the detection of tumor cells in the midst of a mass of normal (*e.g.*, non-transformed) cells through determining the temperature of the cells. In a preferred embodiment of this type the living tissue is obtained from a tumor biopsy. In a related embodiment, the present invention provides a method of detecting the presence of abnormal metabolism of a cell surrounded by healthy tissue (in the midst of a mass of normal cells) comprising detecting the temperature of the cell by placing the cell on a metabolic probe of the present invention (as described above), exciting the fluorophore of the metabolic probe with the appropriate ultraviolet, visible, or infrared light, and detecting the fluorescent signal. A difference in temperature of the cell relative to a control cell, is indicative that the cell is undergoing abnormal metabolism. In one such embodiment the abnormal metabolism of the cell is indicative of a cell having ultraviolet damage. In another embodiment the abnormal metabolism of the cell is indicative of the cell undergoing cachexia. In a third embodiment the abnormal metabolism of the cell is indicative of the cell undergoing apoptosis.

In one particular embodiment of the present invention the metabolic probe is placed on a microscope, the fluorophore is excited,and its emission is detected using the microscope. In one such embodiment, the microscope is a laser scanning confocal microscope that is equipped with an ultraviolet laser for excitation. In another such embodiment the microscope is a fluorescent microscope equipped with a Hg/Xenon lamp with a ultraviolet excitation filter, and a visible emission filter of 615 nm +/- 10 nm.

The present invention also includes a method of making the metabolic probes of the present invention. One such embodiment comprises contacting a polymer with a temperature-sensitive fluorophore in a miscible solvent, wherein a solution of the fluorophore and the polymer is formed. The solution is next placed on the solid substrate, and the polymer is then annealed to the solid substrate.

The solid substrate used in this method is preferably a glass or a plastic. In a more preferred embodiment of this method, the solid substrate is a glass cover slip. In a preferred embodiment of this method, the glass cover slip is immersed in strong acid (*e.g.* 70% nitric acid) prior to placing the polymer-fluorophore on the glass slide. In another embodiment of this method, the solid substrate is rotated during the annealing of a polymer to the solid substrate.

In yet another embodiment of this method, the polymer used is poly(methyl methacrylate). In still another embodiment of this method, the temperature-sensitive fluorophore is $Eu(TTA)_3$. In yet another embodiment the solvent is nitroethane.

In a particular embodiment of this type the metabolic probe is made by a method comprising contacting a solution of 0.1 mM poly(methyl methacrylate) in 96% nitroethane with 200 mM $Eu(TTA)_3$ in 96% nitroethane. The fluorophore/polymer solution is then placed on an acid-washed glass slide (which had been previously immersed in 70% nitric acid, washed with distilled water, rinsed with ethanol, and then dried in a vacuum oven). The glass slide is then mounted on a rotor and rotated. The glass slide is next baked at 100°C for one hour in a vacuum oven to anneal the polymer.

These and other aspects of the present invention will be better appreciated by reference to the following drawings and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the change in fluorescence intensity with temperature for $Eu(TTA)_3$/PPMA. The excitation wavelength was 365nm and the emission wavelength was 621nm.

Figure 2A and 2C are cells as described in the Example viewed by absorbance spectroscopy.

Figures 2B and 2D represent the fluorescence difference spectra obtained from the cells of Figures 2A and 2C respectively, after the addition of FCCP. The cells were excited at 355 nm and the emission was monitored at 614 nm.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for using a metabolic probe as described herein to monitor the temperature and/or metabolic changes of a single cell or group of cells. The cell can either be dissociated from other cells or part of a cell sample. When the cell is a tumor cell such monitoring aids in the understanding of the cell biology of tumor cells. The metabolic probes and related methodology of the present invention may also be used to determine whether tumor cells are present in a fresh biopsy of living tissue in a time frame which would allow the diagnosis to occur during the tumor biopsy. In this manner, the analysis of the biopsy could be made immediately following the removal of the tissue, allowing the health practitioner to immediately make a decision on whether additional tissue must be removed, or alternatively determine that no further surgery is required. Such a procedure has many advantages over current technology which requires an initial biopsy, then typically a 7 to 10 day assessment of the tissue, which many times is followed by an additional surgical procedure. Thus, the present invention allows the medical practitioner to perform one surgical procedure and to immediately react to the tissue analysis thereby preventing further deterioration of the tissue during the time span of the analysis.

The present invention also includes methods of detecting the temperature of a cell using the metabolic probes of the present invention. The metabolic probes of the present invention comprise a solid substrate containing a temperature-sensitive fluorophore which has been embedded in a polymer. When the fluorophore is excited

with the appropriate ultraviolet, visible, or infrared light, it emits a detectable fluorescent signal. This fluorescent signal is effected by the metabolic rate of a cell that is placed on the metabolic probe. Thus, by exciting the fluorophore with the appropriate light and then detecting the intensity of the fluorescent signal, the temperature of the cell can be determined.

Therefore, if appearing herein, the following terms shall have the definitions set out below:

As used herein, a "metabolic probe" comprises a solid substrate containing a temperature-sensitive fluorophore embedded in a polymer, wherein, when the fluorophore is excited with the appropriate wavelength of light (*e.g.* ultraviolet, visible or infrared) the fluorophore emits a detectable fluorescent signal, and wherein, when a living cell (alone, or as part of a cell sample) is placed on the metabolic probe, the fluorescent signal is potentiated (*i.e.*, increases or decreases) due to the heat emitted by the cell (*e.g.*, as measured as the temperature and/or metabolic state of the cell).

As used herein, a "solid substrate" can be made of any solid material, preferably a plastic, metal, or glass, and more preferably a glass lens.

As used herein "about pH 7.5" includes pH 7.0 to pH 8.0.

In one application of the present invention, the temperature is determined of a cell obtained from a cell sample from a patient suspected of suffering from cachexia is determined. Cachexia is a disease state that wastes away the body, and often is fatal. Cachexia, which is triggered by tumor necrosis factor, is associated with increased production of lactic acid and is believed to be the result of activating a futile substrate cycle between fructose 6-phosphate and fructose 1,6-bisphosphate. This burns up cellular ATP [Zentella *et al., Cytokine,* 5:436-447 (1993)]. Thus, a determination of

an increased temperature for cells obtained from a patient suspected of having cachexia is consistent with the diagnosis of cachexia.

In another aspect of the present invention, the mechanism of induced fever by macrophage inflammatory protein can be probed. Fever can be elicited in animals by a factor that is released by macrophages: macrophage inflammatory protein [Myers *et al., Neurochemical Research,* 18:667-673 (1993)]. While it is known that this factor modulates food intake by the animal, it is not yet known where or how they have their effects on cells to increase metabolism and increase temperature. Thus, determining which particular cells (or cell types) are emitting the excess heat causing the fever can be accomplished by detecting the temperature of the individual cells (or cell type).

In a particular aspect of the present invention, the temperature of cultured cells are determined. In one such embodiment, populations of breast tumor cells are used. In another such embodiment, drug-resistant breast tumor cells are used. In yet another embodiment, non-transformed breast cells are used. In a preferred embodiment, two of these cell-types are used. In yet a more preferred embodiment, all three cell types are used. In a preferred embodiment, the breast tumor cells are from a human source. In one particular embodiment, brown adipose tissue cells from tumor cell line HIB 1B are used.

In another embodiment of this aspect of the invention, a slice of breast tissue is used as the cell source. A slice through the breast includes fat cells (which are filled with large fat globules that have very low metabolic levels) and duct cells which are actively metabolizing and secreting. The basal metabolic rate of tumor cells is significantly higher than that of "normal" cells. Assays using surface thermometry of skin tumors indicate a temperature difference (upon illumination) of up to 3.3°C.

In current medical practice, excised tissue obtained during biopsies is prepared for histological assays taking 7-10 days. Thus, the patient is no longer in the surgery room when the results are determined. Subsequent surgery therefore, often needs to

be scheduled. The method of detecting the temperature of cells in the slices using the methodology of the present invention, allows the cells of the tissue slices to be immediately assayed using a standard fluorescence microscope to determine if there are localized "hot spots" of metabolic activity (*i.e.* cells emitting greater that normal quantities of heat) as a fingerprint for tumor cells. Such determinations allow the surgeon to immediately decide whether or not to excise additional tissue.

Still another aspect of the invention includes determining the temperature/metabolism of individual cells, to monitor the shift of quiescent multi-drug resistant cells to the more malignant state. In this case an increase in temperature would be consistent with such a shift. Such monitoring might be particularly useful during chemotherapy, and more particularly, after the end of such treatment.

In a particular embodiment, a cell or tissue biopsy is placed on a metabolic probe and then detected with a laser scanning confocal microscope that is equipped with an ultraviolet laser for excitation which allows simultaneous emission measurements from the temperature-sensitive fluorophore. In another such embodiment the detection is performed on a fluorescence microscope equipped with a Hg/Xenon lamp with an ultraviolet excitation filter and visible emission filter.

As mentioned above, one preferred fluorophore is $Eu(TTA)_3$ which has an excitation maximum of 360 nm and has an emission maximum of 614 nm. More preferably $Eu(TTA)_3$ is combined with the polymer poly(methyl methacrylate), (PMMA), and annealed to a solid substrate to form a metabolic probe. However, other suitable combinations are envisioned by the present invention. Such potential combinations are exemplified in Table 1. These and other combinations can be tested to determine their suitability to serve as a components of the metabolic probe. In particular, the key criteria for choosing a fluorophore compound are that the fluorophore: (i) show a maximal change of fluorescence with change in temperature at 37° C; (ii) have the ability to be embedded in a polymer and away from the aqueous solution; and (iii) be non-toxic to living cells. Thus, such testing can include determining the sensitivity of

the fluorophore to temperature changes at 37°C, the stability of the fluorophore in an aqueous environment, the stability of the fluorophore during embedding into the polymer, and the resistance of the fluorophore to photobleaching (such resistance is required for long-term observations and subsequent calibrations). The greater the fluorophore's sensitivity to temperature change at 37°C, the greater its stability under the above-mentioned conditions, including resistance to the photobleaching, the more suitable is the fluorophore as a component of the metabolic probe. Similarly, the polymer is selected for having an optimizing effect on the temperature-sensitive fluorophore. Finally, the fluorophore/polymer combination must be compatible with the living cells, *i.e.*, they should not adversely affect cell viability. In the Example below, these and/or other factors are described.

The present invention may be better understood by reference to the following non-limiting Example, which is provided as exemplary of the invention. The following example is presented in order to more fully illustrate the preferred embodiments of the invention. It should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE

Determination of Appropriate Fluorophores and Polymers for a Metabolic Probe

Introduction

Several criteria have been introduced in order to select an appropriate fluorophore and polymer for the metabolic probes of the present invention. For example, the sensitivity of the fluorophore to temperature changes at 37°C is an important criteria, with greater sensitivity being desirable. Similarly, the fluorophore is selected for its stability in an aqueous environment, since a viable cell is most stable in an aqueous environment. In addition, a fluorophore is selected for its stability during its embedding into a polymer. For example, the cell samples are to be separated by approximately a 50 nm thickness of polymer from the fluorophore to ensure that

changes of fluorescence are the consequence of temperature emission from the cell, and not secretion of a protein, metabolite, or ion, that could modify the properties of the fluorophore.

Further, a polymer is selected which optimizes the properties of our temperature-sensitive fluorophore. Such a polymer should be selected for relative resistance to photobleaching (a property required for long-term observations and subsequent calibrations). Further a polymer, and the fluorophore should be compatible with living cells so as to ensure that the polymer and fluorophore do not adversely affect cell viability.

Materials and Methods

Coating of coverslips with temperature-sensitive fluorophores

Materials

Dye: Europium Thenoyl trifluoroacetonate, abbreviated $Eu(TTA)_3$ or EuTTA (Advanced Materials, New Hill, NC)

Polymer: Poly (methyl methacrylate), abbreviated PMMA (Aldrich, Milwaukee, WI 18,226-5)

Solvent: 96% Nitroethane (Aldrich, Milwaukee, WI 13.020-6)

Mixing of dye/polymer

The standard polymer (PMMA) concentration in solvent (nitroethane) was 0.1mM (100 mg PMMA in 5 ml nitroethane). Various concentrations of $Eu(TTA)_3$ were used. The best fluorescent signal was with the 200 mM $Eu(TTA)_3$ in solvent (100 mg of EuTTA in 5 ml Nitroethane). The dye/polymer mixture was stirred with a magnetic stirrer until completely dissolved and then filtered through a 0.45µm pore filter (Gelman Sciences, Acrodisc 13CR PTFE) leaving a completely clear solution. To minimize problems with hydration, the dye and polymer was stored under N_2, and the solvent was stored with molecular sieves.

The thickness of the layer can be adjusted by varying the polymer concentration (the higher the thicker), and the spinning speed (the slower the thicker).

Preparation of cover slips

All cover slips were acid washed in nitric acid. The cover slips were immersed in 70% nitric acid for 5 minutes, rinsed with dH_2O, rinsed with 95% Ethanol, and then dried in vacuum oven at 100°C. The acid washing was required for the polymer-fluorophore to remain anchored firm and stable to the glass.

Spin coating process. The cover glass slide was mounted on a rotor and covered with the polymer/fluorophore/solvent sample. The cover slip was rotated with a typical speed of 600 rpm for 1 minute. The cover slip was then baked at 100°C for one hour in a vacuum oven to anneal the polymer.

Characterization of coatings. The coatings on the cover slips were characterized by several methods. The homogeneity was assayed under phase contrast microscope using a 20x or 40x objective. The homogeneity was also assayed under a fluorescent microscope (Nikon Diaphot) using a 40x objective or on a laser scanning confocal microscope (Ultima, Meridian Instruments, Okemos, MI) using a 60x objective. The absorption of the coated cover slips was assayed at 365 nm with a diode-array spectrophotometer (Milton Roy Spectronic, Array 3000). The temperature-sensitivity of various mixtures of fluorophores, polymers, solvents was initially tested in temperature controlled cuvette in a fluorescence spectrofluorimeter (Aminco Bowman 2, SLM Aminco). Cover slips were cut and fitted at a 45 degree angle into the water filled cuvettes. This configuration was used for determination of:

- optimal excitation and emission spectrum by recording excitation and emission scans;
- photobleaching rate and stability of layer in water (tested by recording the average fluorescent signal over time); and

- temperature coefficient (change of intensity per degree) which was determined by recording fluorescent intensity changes during temperature changes of normally 1°C.

Cell growth on coverslips

The human breast tumor line MCF-7 cells were grown on the coatings. The growth rate on the coatings was a little slower than on the polystyrene surface of the culture dishes, but the viability was higher than 80%.

Cell culture

The MCF-7/ADR cells are a cell line resistant to chemotherapeutics which is derived from a human breast carcinoma. They are maintained in Modified Eagle's medium with phenol red, Bovine insulin 10 μg/mL and L-glutamine and 10% FBS in a humidified incubator at 37°C and 5% pCO_2 (Forma Scientific, OH). In addition, the MCF-7/ADR cells are continuously maintained in 0.8 μM Adriamycin.

Cells are observed in an Olympus fluorescence microscope equipped with a Xenon lamp, a Uniblitz shutter (Vincent and Associates, Rochester NY) and filter wheels on the excitation and emission side. The shuttering of the light source is controlled with a computer. A filter holder was manufactured to hold 450 nm and 490 nm excitation filters. The date are collected on a Hamamatsu 4972 cooled charged coupled device (Hamamatsu Photonics) and digitized with software written in National Instruments Lab View. Cells are visualized in a temperature controlled chamber with a constant perfusion of media.

The cells are excited with a λex of 355 nm and λem of 614. When the ratio was taken of successive images, the cells could not be detected. However, upon inclusion of FCCP, an proton-ionophore which increases the proton permeability of the mitochondria and thus increases the hydrolysis of ATP, there is an increased fluorescent emission consistent with an increase of temperature. The greatest increase

of temperature is approximately 100 mK and it is most consistently observed in the growth cone of the cell (Figures 2B and 2D).

Fluorescence measurements

The fluorescence measurements were performed with a fluorescence microscope equipped with a Hg/Xenon lamp using an ultraviolet excitation filter and emission filter. $Eu(TTA)_3$ excites at 360 nm and emits at 614 nm.

Results

Choice of fluorescent compound: It was necessary to eliminate all water in the solvent and polymer before coating the cover slips. Unfortunately water adversely affected the stability of the fluorophore/polymer mix and many of the commonly used reagents to dehydrate the solvent also adversely affected the fluorophores. One key was to use a fluorophore that could be treated with dehydrating reagents without adverse effects. It turned out that one of the potential fluorophores (Eu-FOD) would undergo major changes in properties when we baked it onto the glass cover slips. $Eu(TTA)_3$ did not have this problem and was therefore selected as a good fluorophore.

Choice of polymer: The fluorophores being studied change their fluorescence in response to temperature. However, their fluorescence could also be sensitive to particular proteins, carbohydrates, pH or ionic changes. So the fluorophore is being embedded in a polymer that will be used to coat coverslips. Polystyrene was our first choice for a support polymer. It is stable to many different treatments, it is non-toxic, it can be used as a substrate for growing cells. Unfortunately, the response properties of EuTTA to temperature were weak in the polystyrene. It appears as if the benzene rings in the polystyrene adversely interacted with the groups in the EuTTA resulting in a loss of activity. On the other hand, PMMA is a non-aromatic fluorophore. This polymer had been known to be sensitive to the UV wavelengths used to excite EU-TTA. Indeed, UV light is often used to etch PMMA. However, after varying the concentrations of PMMA: EuTTA: solvent: dehydration agent, a mixture of EuTTA: PMMA that is very stable on coverslips was determined. This mixture can be

immersed in a water environment without adverse effects on mechanical stability; its fluorescent signal is stable for at least an hour during UV excitation; water does not adversely affect the fluorescent signal; and its fluorescence changes 2.5% for every one degree change centigrade. Furthermore, the noise is very low (approximately 100:1) allowing the resolution of changes of 0.01 °C. A plot of the change in fluorescence intensity with temperature is depicted in Figure 1. In Table 1, the $Eu(TTA)_3$/PMMA mixture is compared with a sampling of other fluorophore/polymer couples in regard to their maximum Log Slope/°C.

Table 1

Fluorophore	Polymer	Maximum Log Slope/°C	Temp
Anthracene	Cellulose Acetate		
Coumarin	PMMA	-0.004	60
CuOEP	GP-197	-0.0113	-100
Erythrosin B	Polycarbonate		
Europium thenoyltrifluoroacetonate (EuTTA)	model airplane dope	-0.036	0
Europium thenoyltrifluoroacetonate (EuTTA)	PMMA	-0.049	
Europium thenoyltrifluoroacetonate (EuTTA)	marathon	-0.0095	
HC-295	GP-197		
La_2O_2S:Eu(1%)	solid	-0.0031	-150
Perylene	model airplane dope	-0.0134	25
Perylenedicarboximide (PDC)	PMMA	-0.007	75
Perylenedicarboximide (PDC)	SOA	-0.06	75
PtOEP	GP-197	-0.0033	10
Pyrene	Cellulose Acetate		
Pyronin B	PMMA	-0.046	60
Pyronin Y	model airplane dope	-0.055	50
Pyronin Y	PMMA	-0.072	75
Pyronin Y	Polycarbonate	-0.0168	50

Fluorophore	Polymer	Maximum Log Slope/°C	Temp
Quinizarin	Polystyrene	-0.084	90
Rhodamine B	Cellulose Acetate	-0.0167	-125
Rhodamine B	PVC	-0.014	-5
Rhodamine B	PVP	+0.057	-30
Rhodamine B	Polyurethane	-0.0175	80
Rhodamine B	model airplane dope	-0.018	
Rose Bengal	Ethyl Cellulose	-0.09	80
Rubrene	PMMA	-0.034	5
Rubrene	SOA		
Ruthenium Comp. DJ-171	GP-197		
Ruthenium Comp. DJ-201	GP-197	-0.042	0
Ruthenium Comp. DJ-275	GP-197	-0.018	0
Ruthenium Comp. VG-220-1-2	GP-197	-0.0573	10
Ruthenium Comp. VG-225-2	GP-197	-0.0212	-10
Ruthenium Comp. VH-127	GP-197	-0.0071	-150
Ruthenium Comp. SC-324	GP-197	-0.0233	0
Ruthenium Comp. SC-393	GP-197	-0.0343	0
Ruthenium (bpy)/Zeolite	PVA	-0.0269	40
Ruthenium (trpy)	Ethyl Cellulose	-0.0131	-140
Ruthenium (trpy)	GP-197	-0.0134	-140
Ruthenium (trpy)	model airplane dope		
Ruthenium (trpy)	PMMA		
Ruthenium (trpy)/Zeolite	PVA	-0.0096	-100
Sulphorhodamine B	model airplane dope	-0.0375	105
TTMHD	solid	-0.0293	25

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing

description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes , and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in there entireties.

WHAT IS CLAIMED IS:

1. A method of detecting the temperature of a cell comprising:

(a) placing a cell on a metabolic probe comprising a solid substrate containing a temperature-sensitive fluorophore embedded in a polymer, wherein when the fluorophore is excited with the appropriate ultraviolet, visible or infrared light it emits a detectable fluorescent signal; and wherein when a living cell is placed on the metabolic probe, the fluorescent signal is effected by the metabolic rate of the cell;

(b) exciting the fluorophore with the appropriate ultraviolet, visible, or infrared light; and

(c) detecting the fluorescent signal, wherein the intensity of fluorescent signal detected is indicative of the temperature of the cell.

2. The method of Claim 1 wherein the polymer is a non-aromatic synthetic polymer.

3. The method of Claim 1 wherein the fluorophore can emit a stable fluorescent signal while embedded in a polymer adjacent to an aqueous solution for at least one hour upon excitation with ultraviolet light at 37 degrees Celsius, pH 7.5.

4. The method of Claim 2 wherein the polymer is poly(methyl methacrylate).

5. The method of Claim 4 wherein the fluorophore is $Eu(TTA)_3$.

6. A method of detecting the metabolic activity of a cell comprising detecting the temperature of the cell by the method of Claim 1 and correlating the temperature with its metabolic activity.

7. The method of Claim 6 wherein the cell is a tumor cell.

8. A method of detecting the presence of abnormal metabolism of a cell surrounded by healthy tissue comprising detecting the temperature of the cell by the method of Claim 1, wherein a difference in temperature of the cell relative to a control cell, is indicative that the cell is undergoing abnormal metabolism.

9. The method of Claim 8 wherein the living tissue is obtained from a tumor biopsy.

10. The method of Claim 8 wherein the abnormal metabolism of the cell is indicative of ultraviolet damage, cachexia, or apoptosis.

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Application No: GB 9900118.2 **Examiner:** Michael R. Wendt
Claims searched: All **Date of search:** 19 March 1999

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.Q): G1B (BBG)

Int Cl (Ed.6): G01N 21/64, 33/50; C12N 5/06, 5/08

Other: EPODOC, PAJ, WPI, CAS

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
Y	Photochem. & Photobiol. Vol. 62, No. 3, 1995, pages 416 - 425. Article by Chapman, Liu et al. e.g. see the Abstract.	1
Y	Int. J. Radiation Oncology Biol. Phys. Vol. 16, 1989, pages 1565 - 1570. Article by P. Olive. e.g. see the Abstract.	1 & 8 - 10
Y	Applied Phys. Letters Vol. 40(9), 1982, pages 782 - 784. Article by Kolodner. * mentioned in application* e.g. see the Abstract.	2, 4 & 5

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.